

Appl. No. 09/998,822
Amendment Dated: November 7, 2003
Reply to Office Action of Aug. 25, 2003
Docket No.: MSU 4.1-542

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning on page 26, line 37 with the following amended paragraph:

-An alternate method for screening hybridomas for antibody production is as follows. *Pythium insidiosum* is heat-denatured in 0.5 M Tris (pH 7.4) with 10% SDS, 20% glycerol and 5% 2-mercaptoethanol. The denatured antigens are separated by SDS-polyacrylamide gel electrophoresis in a 12-20% (v/v) linear gradient gel with a 4% (v/v) stacking gel. The separated antigens are electrophoretically transferred to Western PVDF membranes at 100 volts for 1.5 hours, then 150 volts for 0.5 hours. The membranes are then blocked overnight in 1% by volume bovine serum albumen in 0.5% Tween-Tris TWEEN TRIS buffered saline (Blocking buffer). The blots are air-dried and stored frozen. Prior to use, the membranes are incubated with bovine serum albumin in Blocking buffer at a range of 1:10 to 1:100 ratio for two hours. Afterwards, the membranes are washed in 0.5% Tween-Tris TWEEN TRIS buffered saline and then incubated with monoclonal antibodies from the various hybridoma clones. The membranes are developed as disclosed in the prior art, e.g., Granstrom et al., J. Vet. Diag. Invest. 5: 88-90 (1993) or *Antibodies, A Laboratory Manual*, eds. Harlow and Lane, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988).-

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Please replace the paragraph beginning on page 34, line 31 with the following amended paragraph:

-The ELISA was carried out as per Mendoza et al. (Mendoza et al., Clin. Diag. Lab. Immunol. 4: 715-718 (1997)). Flat-bottom polystyrene microtiter plates (96-well ~~Immulon~~ IMMULON; Dynatech Laboratories Inc., McLean, Virginia) were coated with the antigen prepared as above and incubated overnight at 4° C and then blocked for 1 h at 37° C with 5% gelatin. Dilutions of the sera under investigation were prepared and then added to the coated plates and incubated for 1 h. After several washes, 100 µl of a horseradish peroxidase-conjugated rabbit anti-horse, anti-dog, or anti-cat (heavy and light chains) antibody was added to each well and then incubated at 30° C for 1 h. After incubation, the reaction was stopped with chromogen buffer and color development was recorded in a Dynatech MR 5000 ELISA plate reader at 490 nm. The immunoperoxidase assay performed on sera from two of the dogs was not carried out in our facilities, but was carried out in other laboratories at the request of their owners.-

Please replace the paragraph beginning on page 53, line 7 with the following amended paragraph:

-To evaluate the different IgG isotypes triggered in the experimentally-induced pythiosis or by

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the PIV, and IgG isotype assay is performed. Rabbits are bled before inducing pythiosis, 14 days post-vaccination, and 14 days after the second immunization. The isotype assays measure the total immunoglobulin populations in the rabbit. briefly, 50 μ l of the PIV (2 mg/ml) is coated on flat-bottomed polystyrene microtiter plates (96-well, ~~Immunion~~ 2 IMMUNION2, Dynatech Laboratories, Inc., Virginia) at 4° C for 24 hr. The plates are then reacted against the rabbit sera as per Mendoza et al., Clin. Diagn. Lab. Immunol. 4: 715-718 (1997) followed by reacting with anti-IgG isotypes (IgG₁, IgG₂, and IgG₃) according to the manufacturer's instructions (Accurate Chemicals, New York). In addition, to monitor the Th2 to Th1 switching, IgE levels in all the rabbits are determined. The immunological data provided by the method provides a means for validating the Th2 to Th1 switching hypothesis which can be extrapolated to other infectious diseases of animals. -

Please replace Table 1 beginning on page 38 with the following amended Table 1:

Table 1. Clinical features of equine cases with pythiosis used in this study and their responses to *Pythium insidiosum*-vaccine

State	Age/sex	Lesions	Duration of illness	Diagnosis	Previous treatments	Vaccination reaction	Outcome
AR (Gi)	4 y/F	Abdomen, 220X220 mm	4 months	ID, ELISA (+)	Surgery, drugs	Strong, 123 mm	Cured
FL (Sc)	13 y/F	Face, 80X30 mm	>4 months	ID, ELISA (+)	Surgery, Drugs	Mild, 25 mm	Cured
FL (Sn)	12 y/M	Limb, 150X100 mm	>2 months	ID (+), clinical	Surgery	Mild, 60 mm	Cured
FL (Jo)	20 y/M	Limb, 300X150 mm	>2 months	ID (+), clinical	Several surgeries	Mild, 60 mm	Cured
FL (Wa)	8 y/F	Limb, 60X50 mm	5-7 days	ID (+), Clinical	Surgical debridement	Strong, 100 mm	Cured
FL (Ho)	5 y/F	Shoulder & abdomen 50X50 & 120X20mm	15 days	ELISA (+)	Cryosurgery	Strong, 200 mm	Cured
FL (Pe)	22 y/M	Limb, 60X40	17 days	ID, ELISA (+)	Surgical debridement	Mild, 30 mm	Cured
LA (Re)	5 y/M	Limb, 120X100 mm (two lesions) Limb, 250X250 mm	2 months	ELISA (+)	Surgical debridement	Mild, 90 mm	Cured
MS (Ba)	3 y/F		>2 months	ELISA (+)	Topical drugs	Strong, 150 mm	Cured
MS (Pa)	15 y/F	Limb, 300X200 mm	4 months	ELISA (+)	Topical drugs	Strong, 170 mm	Cured
MS (Im)	2 y/M	Limb, 300X300 mm	4 months	ELISA (+)	Topical drugs	Strong, 200 mm	(Vaccine + surgery) Cured
MS (So)	7 y/F	Limb, 240X240 mm	3 months	ELISA (+), Histopathology (+)	Topical Drugs	Strong, 150 mm	(Vaccine + surgery) sacrificed (+)
NC (Sa)	20 y/M	Inguinal, 60X50 mm (two lesions)	>2 months	ID (+), ELISA (+)	Surgery	Mild, 40 mm	Not cured
NC (Ga)	14 y/F	Inguinal 200X150 mm	>2 months	ID (+), ELISA (+)	Surgery	Mild, 30 mm	Not cured
TN (Re)	4 y/F	Abdomen, 200X80 mm	2 months	ELISA (+)	Topical Drugs	No response	Not cured
TX (Ta)	13 y/M	Limb, 250X100 mm	>4 months	ELISA (+)	Topical Drugs	Strong, 200 mm	Died
TX (Ah)	5y/M	Limb, 280X210 mm	1 month	Clinical, kunkers ELISA (+)	Surgery, drugs	Mild, 30 mm	Cured
TX (Co)	6 y/F	Limb, 100X80 mm	1 month	ELISA (+)	Topical drugs§	Weak, 15 mm	Not cured
TX (Sn)	22 y/M	Mouth, 150X100 mm	>2 months	ELISA (+)	Surgical debridement Histopathology (+)	Weak, 5 mm	Not cured